

### Remarks

The specification of the present application was amended to cross-reference other applications relied upon for priority. All such previous applications are hereby incorporated by reference. In addition, the claims originally filed have now been cancelled and replaced with new claims 11-22. The new claims are fully supported by the claims that have been canceled in the specification. Since these amendments do not add new matter to the application, their entry is respectfully requested.

### Conclusion

In light of the amendments made herein, Applicants believe that the present application is now in condition for allowance. Early notice to this effect is earnestly solicited.

If, in the opinion of the Examiner, a phone call may help to expedite the prosecution of this application, the Examiner is invited to call Applicants' undersigned attorney at (202) 419-7013.

Respectfully submitted,

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**10/526389**

DT06 Rec'd PCT/PTO 0 2 MAR 2005

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| Intl. Appl. No.:   | PCT/EP2003/008631  |
| Intl. Filing Date: | August 5, 2003   |
| For:               | <b>Use of Malate Dehydrogenase for NADH<br/>Regeneration</b> |
| Inventor(s):       | Náamnieh, et al.   |
| Your Ref:          | 020365 OC  |
| Our Ref:           | 7601/84242   |

**“MARKED-UP” VERSION OF SUBSTITUTE SPECIFICATION**

## Use of ~~malate dehydrogenase~~ Malate Dehydrogenase for NADH ~~regeneration~~ Regeneration

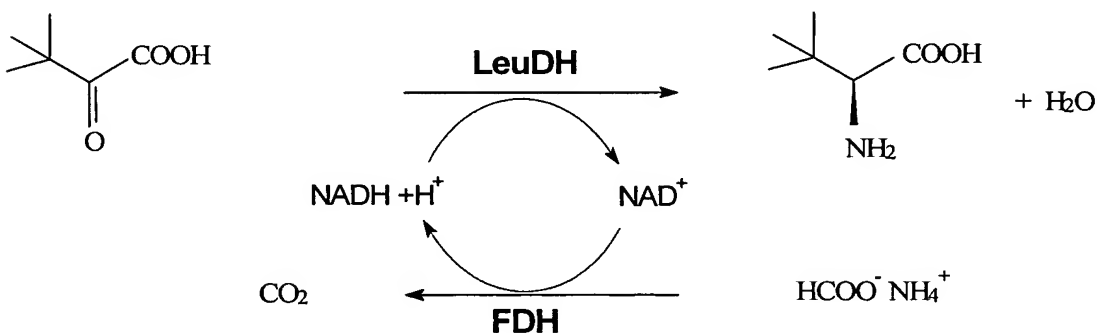
### Field of the Invention

The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the ~~present~~ invention relates to an ~~enzymatically operating process, in which, in~~ a coupled enzymatic reaction system, in which NAD(P)H is consumed by one enzyme for the preparation of the organic compound and the NAD(P)H is simultaneously regenerated by a second enzyme system. The invention also includes A a reaction system which operates ~~according to the invention~~ in this manner and an advantageous whole cell catalyst catalysts or suitable plasmids ~~are also proposed~~.

### Background of the Invention

The production of optically active organic compounds, ~~e.g. alcohols and amino acids,~~ by a biocatalytic route is increasingly gaining importance. The coupled use of two dehydrogenases with cofactor regeneration has emerged, inter alia, as a route for the large-scale industrial synthesis of these compounds, in particular alcohols and amino acids (DE19753350, EP118750).

Equation 1:



Equation 1 shows the ~~in situ~~ *in situ* regeneration of NADH with the NAD-dependent formate dehydrogenase from *Candida boidinii* in the reductive amination of trimethyl pyruvate to give L-tert-leucine (Bommarius ~~et al.~~ *et al.*, *Tetrahedron Asymmetry* 1995, 6:2851-2888(1995)).

In addition to their catalytic ~~property~~ properties and efficiency, ~~the~~ biocatalysts ~~efficiently employed in an aqueous medium furthermore~~ have the advantage that, in contrast to a large number of synthetic metal-containing catalysts, the use of toxic metal-containing starting substances, ~~in particular those which contain heavy metals and are therefore toxic,~~  
 5 can be dispensed with. The use of expensive and ~~furthermore~~ hazardous reducing agents, ~~e.g. e.g., borane, in the case of asymmetric reduction~~ can also be dispensed with.

The FDH, ~~e.g. e.g.,~~ from *Candida boidinii*, that has been successfully employed to date in these systems has the disadvantage that the specific activity of this enzyme class at  
 10 4-8 U/mg is very low. This necessitates the use of a large amount of expensive enzyme with recycling. As a result it is difficult to perform processes on an industrial scale economically ~~which is difficult from the apparatus point of view if a process designed in this way is to be carried out advantageously under economic aspects on an industrial scale.~~

Malate dehydrogenase (MDH), also called "malic enzyme," catalyses the oxidative decarboxylation of malate to pyruvate. Numerous malate dehydrogenases ~~from various organisms~~ are known, ~~thus, inter alia, from~~ including enzymes from higher animals, plants and microorganisms. A distinction is made between four types of malate dehydrogenases, which are classified into the enzyme classes E.C. 1.1.1.37 to E.C. 1.1.1.40  
 20 (<http://www.genome.ad.jp>). NAD and/or NADP is required as a cofactor, depending on the type of malate dehydrogenase used.

~~On the basis of~~ Due to the irreversibility of the oxidative decarboxylation reaction of L-malic acid to pyruvate, the use of malate dehydrogenase is also appropriate in the systems  
 25 described ~~above in respect of a favourable cofactor regeneration~~. The use of malate dehydrogenase for regeneration of NAD is described, for example, by Suye, et al. ~~et al. in a work from 1992 (S. I. Suye, M. Kawagoe, S. Inuta, Can. J. Chem. Eng. 1992, 70, 306-312 70:306-312 (1992)).~~ NADH regeneration by means of malate dehydrogenase is used ~~here~~ for reductive amination of pyruvate by means of an alanine dehydrogenase, NADH being  
 30 consumed. The pyruvate is formed by oxidative decarboxylation from L-malic acid, and is immediately consumed again in the following step by alanine dehydrogenase. ~~A concentration of pyruvate in the reaction solution is therefore avoided, whereby the~~ The problem of any ~~inhibitions of the enzymes involved by~~ inhibition due to the presence of

~~large~~ amounts of pyruvate ~~in the stoichiometric range is also eliminated~~ is therefore avoided. Nevertheless, the system ~~described~~ is limited exclusively to the production of alanine (see also S. I. Suye, *Recent Res. Devel. Ferment. Bioeng.* ~~1998, 1, 55-64~~ 1:55-64 (1998)).

~~Interestingly in spite of the technically high potential apart from these works by the Suye study group no further works using "malate dehydrogenase" for regeneration of the oxidized cofactor NAD<sup>+</sup> during reductions of ketones or reductive aminations of keto acids have been described.~~

### **Description of the Invention**

The ~~object of the present invention was therefore to provide a further~~ is directed to a process for the preparation of chiral organic compounds, such as amino acids or alcohols, ~~which can be obtained by using~~ a coupled enzymatic malate dehydrogenase reaction system ~~as described above, using malate dehydrogenase, which is not limited to the preparation of one substance. In particular, it~~ It should be possible to employ use this process on an industrial scale ~~particularly advantageously under economic and ecological aspects in a manner that is both economically and ecologically advantageous.~~

~~This object is achieved by a process with the characterizing features of claim 1 of the present invention. Claims 2 to 7 relate to preferred embodiments. Claims 8 and 9 protect a reaction system according to the invention and a correspondingly operating whole cell catalyst. Claim 10 protects preferred plasmids.~~

More specifically, the invention is directed to ~~In~~ a process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system. The system comprising comprises a first enzymatic transformation of an organic substrate, during which NAD(P)H ~~being is~~ consumed, and ~~the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, during which the NAD(P)H is regenerated.~~ The second enzymatic transformation is catalyzed by malate dehydrogenase and involves the ~~with~~ oxidation of L-malic acid to pyruvate and CO<sub>2</sub> ~~CO<sub>2</sub> as a result of not employing the~~ The pyruvate formed from the second enzymatic transformation is not used as the substrate in the first enzymatic transformation, ~~the object described is achieved in an entirely surprising but in return no less advantageous manner. It can be regarded entirely as~~

~~is surprising that~~ Surprisingly, the simultaneous use, e.g. ~~e.g.~~ of an amino acid dehydrogenase with malate dehydrogenase, ~~which has a decarboxylating action,~~ is possible without problems ~~without the occurrence of cross-reactions~~ due to cross-reactivity. In addition, ~~no inhibiting influences of the~~ substrates and products of the primary reaction ~~on~~ do not appear to inhibit the malate dehydrogenase ~~and reaction or~~ vice versa ~~can be~~ detected. In particular, it is a positive feature that the pyruvate formed as the a by-product does not have ~~an inhibiting action on~~ inhibit the malate dehydrogenase itself or ~~on~~ the alcohol or amino acid dehydrogenases employed in parallel.

Enantiomerically enriched alcohols or amino acids ~~are advantageously~~ can be prepared with the process ~~according to~~ of the present the invention. In this case, inexpensive alcohol dehydrogenase or amino acid dehydrogenase ~~which are known universally are possible~~ may be used as enzymes the enzyme for the first enzymatic transformation. The expert is in principle ~~free in the choice thereof, which is made according to the nature of the~~ substrate spectrum, stability and rate of conversion of the enzyme in question. Known Specific enzymes of this origin that may be used are described ~~in K.~~ by Drauz, H. Waldmann (eds.), *et al.* (Enzyme Catalysis in Organic Synthesis, volume III, Wiley-VCH, Weinheim, **2002**, chapter 15, (2002)). The use of the alcohol dehydrogenase from either the organisms organism *Rhodococcus erythropolis* (S-ADH) ~~or~~ *Lactobacillus kefir* (R-ADH) (ADH from *R. erythropolis*: J. (Peters, T. Zelinski, M. R. Kula *et al.*, Purification and characterization of a novel carbonyl reductase isolated from *Rhodococcus erythropolis*, *J. Biotechnol.* 1994, 33, 283-292 *J. Biotechnol.* 33:283-292 (1994)) ~~or~~ *Lactobacillus kefir* (R-ADH) (ADH from *Lactobacillus kefir*: C. W. Bradshaw, W. Hummel, C. H. Wong, *Lactobacillus kefir* Alcohol Dehydrogenase: A Useful Catalyst for Synthesis, *J. Org. Chem.* 1992, 57, 1532-1536. *et al.*, *J. Org. Chem.* 57:1532-1536 (1992)) is particularly advantageous especially preferred. In respect of preferred Preferred amino acid dehydrogenases, ~~the expert is referred to,~~ for example, leucine dehydrogenases or phenylalanine dehydrogenases, are described by (A. Bommarius in: Enzyme Catalysis in Organic Synthesis (eds.: K. Drauz, H. Waldmann), volume III, Wiley-VCH, Weinheim, **2002**, chapter 15.3, (2002)).

Malate dehydrogenases are also well known in the art. ~~familiar to the expert (lit. see above or the dissertation by S. Naamnich, University of Düsseldorf, in preparation).~~ Here

also, the expert will choose the dehydrogenase which can be employed most efficiently for his purpose. In principle, those malate dehydrogenases which regenerate the NAD(P)H in to an extent such that ~~no~~ a bottleneck does not arise ~~arises for~~ during the course of the reaction of the other enzyme should be employed. The ~~known~~ malate dehydrogenase from *E. coli* K12 is preferred in this connection. ~~Gene isolation and cloning are described in S. Naamnieh's dissertation, University of Düsseldorf, in preparation p. 70 et seq.~~

In principle, the process according to the invention can be carried out in purely aqueous solution. However, it is also possible to add ~~any desired parts of~~ a water-soluble organic solvent to the aqueous solution in order to ~~e.g., for example,~~ optimize the reaction ~~in respect of~~ for poorly water-soluble substrates. Possible Examples of such solvents are, in particular, include ethylene glycol, DME ~~or~~ and glycerol. ~~However, multi-phase~~ Multi-phase, in particular two-phase, systems comprising an aqueous phase can ~~furthermore~~ also serve as the solvent mixture for the process ~~according to the invention~~. The use of certain solvents which are not water-soluble has ~~already proved itself here (DE10233107)~~ been described in DE10233107 and the The statements made ~~there in this respect~~ therein also apply ~~here accordingly~~ to the present process.

~~The expert in principle has a free~~ One of skill in the art will choose ~~choice of the~~ temperature present during the reaction, ~~He preferably directs himself towards~~ with the objective of obtaining a the highest possible yield of product, in the highest possible purity, in the shortest possible time. ~~Furthermore, the~~ The enzymes employed should be sufficiently stable under the ~~temperatures employed~~ temperature chosen and the reaction should proceed with the highest possible enantioselectivity. ~~In respect of the use of~~ When using enzymes from thermophilic organisms, it is entirely possible for temperatures of 100°C to represent the upper limit of the temperature range in the reaction. A temperature of -15°C is ~~certainly~~ appropriate as the lower limit in aqueous systems. A In general, a temperature interval between 10 and 60, particularly and preferably between 20 and 40°C is advantageously to be established will typically be used in reactions.

The pH during the reaction is determined ~~by the expert from the~~ based upon enzyme ~~stabilities~~ stability and conversion rates ~~and is adjusted accordingly for the process according to the invention~~. For the malate dehydrogenase from *E. coli*, it has been found

that the optimum pH is > 10. In general, the range preferred for enzymes will be from pH 5 to 11, ~~is chosen. A pH range more preferably~~ from 5.5 to 10.0, ~~in particular and still more preferably from~~ 6.0 to 9.0, ~~can preferably be present.~~

5        ~~The invention also provides a coupled enzymatic reaction system for the preparation of enantiomerically enriched organic compounds, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO<sub>2</sub>, wherein the pyruvate formed from the~~  
 10 ~~second enzymatic transformation is not employed as the substrate in the first enzymatic transformation. The same advantages and preferred embodiments as have already been mentioned in respect of the process according to the invention apply in principle to this reaction system.~~

15        The reaction system is advantageously employed, for example, in a stirred tank, a cascade of stirred tanks or in membrane reactors, which can be operated both in batch operation and continuously. In the context of the invention, the term "membrane reactor" is understood as meaning any reaction vessel in which the catalyst is enclosed in a reactor, while low molecular weight substances are fed to the reactor or can leave it. The membrane  
 20 ~~here~~ can be integrated directly into the reaction space or incorporated outside in a separate filtration module, in through which the reaction solution flows continuously or intermittently ~~through the filtration module~~ and the retained product is recycled into the reactor. Suitable embodiments are described, ~~inter alia~~ inter alia, in WO98/22415; ~~and in~~ Wandrey et al. et al. (in Yearbook 1998, Verfahrenstechnik und Chemieingenieurwesen, VDI p. 151 et seq. (1998); in Applied Homogeneous Catalysis with Organometallic Compounds, vol. 2, VCH 1996, p. 832 et seq. (1996)); ~~and in~~ Kragl et al., (Angew. Chem. 1996, 6, 684 et seq. Angew Chem. 6:684 et seq (1996)). The continuous procedure which is possible in this apparatus, in addition to the batch and semi-continuous procedure, can be carried out ~~here as desired~~ in the cross-flow filtration mode (fig. 3) or as a dead-end  
 30 filtration (fig. 2). Both process variants are described ~~in principle~~ in the prior art (Engineering Processes for Bioseparations, ed.: L.R. Weatherley, Heinemann, 1994, 135-165 (1994); Wandrey et al. et al., Tetrahedron Asymmetry 1999, 10, 923-928 Tetrahedron Asymmetry 10:923-928 (1999)).



The present invention also provides whole cell catalysts comprising a cloned gene for a first enzyme for transformation of an organic substrate and a cloned gene for a malate dehydrogenase, ~~these being~~ The first enzyme is capable of preparation of an enantiomerically enriched organic compound in a first enzymatic transformation, during  
 5 which NAD(P)H ~~being is~~ consumed, ~~and of allowing the regeneration of the~~ The NAD(P)H is regenerated ~~to take place~~ in a second enzymatic transformation catalyzed by malate dehydrogenase, in which ~~with oxidation of~~ L-malic acid is oxidized to pyruvate and CO<sub>2</sub>, ~~wherein the~~ The pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation. The whole cell catalyst  
 10 according to the invention preferably has an enzyme (polypeptide) with amino acid or alcohol dehydrogenase activity and one with malate dehydrogenase activity, preferably originating ~~in particular~~ from the organisms mentioned above.

Microorganisms which can be used ~~are in principle all the organisms possible to the~~  
 15 ~~expert for this purpose, such as e.g. include:~~ yeasts, such as *Hansenula polymorpha*, *Pichia* sp. and *Saccharomyces cerevisiae*; prokaryotes, such as *E. coli* and *Bacillus subtilis*; ~~or and~~ eukaryotes, such as mammalian cells and insect cells. *E. coli* strains are preferably ~~to be~~ used ~~for this purpose~~. The following are ~~very~~ particularly preferred: *E. coli* XL1 Blue, NM 522, JM101, JM109, JM105, RR1, DH5 $\alpha$ , TOP 10<sup>+</sup> or HB101. An organism as mentioned  
 20 in DE10155928 is preferably employed as the host organism. The advantage of such an organism is the simultaneous expression of both polypeptide systems, which means that only one ~~rec-organism~~ organism has to be cultured for the reaction according to the invention.

25 To coordinate the expression of the polypeptides ~~in respect of their rates of~~ ~~conversion~~, the corresponding coding nucleic acid sequences can be accommodated on different plasmids with different numbers of copies and/or promoters of different potency ~~can be used for an expression of the nucleic acid sequences of different intensity. In~~ Ideally  
in such coordinated enzyme systems, ~~advantageously~~ no accumulation of an intermediate  
 30 compound occurs, and the reaction in question can proceed at an optimum overall rate. ~~However, this is adequately known to the expert~~ These principles are well known in the art (Gellissen, G.; Piontek, M.; Dahlems, U.; Jenzelewski, V.; Gavagan, J. W.; DiCosimo, R.; Anton, D. L.; Janowicz, Z. A. (1996), Recombinant *Hansenula polymorpha* as a biocatalyst.

~~Coexpression of the spinach glycollate oxidase (GO) and the *S. cerevisiae* catalase T (CTT1) gene, Appl. Microbiol. Biotechnol. 46, 46-54 *et al.*, Appl. Microbiol. Biotechnol. 46:46-54 (1996); Farwick, M.; London, M.; Dohmen, J.; Dahlems, U.; Gellissen, G.; Strasser, A. W.; DE19920712).~~

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It is ~~very particularly advantageous~~ preferred that the whole cell catalyst according to the invention ~~optionally~~ further metabolizes the pyruvate formed in the reaction ~~according to the invention, as it may~~ and uses it as a nutrient source. Whole cell catalysts designed in this manner ~~mean~~ have the advantage that the pyruvate is not obtained as a by-product of the reaction and therefore ~~also~~ does not have to be separated off from the chiral product actually desired in further process steps.

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The preparation of the whole cell catalyst can ~~in principle~~ be carried out by ~~measures known to the expert~~ using methods that are well known in the art (Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989) *et al.*, Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York (1989); Balbas, P. and Bolivar, F. (1990), ~~Design and construction of expression plasmid vectors in *E. coli*~~ *et al.*, Methods Enzymol. 185, 14-37 *Meth. Enzymol.* 185:14-37 (1990); Rodriguez, R.L. and Denhardt, D. T *et al.* (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 205-225, Butterworth, Stoneham, 1988)). ~~In~~ With respect to general methodology of molecular biology ~~respect of the general procedure~~ (PCR, cloning, expression etc.) reference may also be made to the following literature ~~and that cited there~~: Universal GenomeWalker™ Kit User Manual, Clontech, 3/2000 and literature cited ~~there~~ therein; Triglia, T.; Peterson, M. G. and Kemp, D.J. (1988), ~~A procedure for in vitro amplification of DNA segments that lie outside the boundaries of known sequences, Nucleic Acids Res. 16, 8186~~ *et al.*, Nucl. Ac. Res. 16:8186 (1988); Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989) *et al.*, Molecular cloning: a laboratory manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York 1989); and Rodriguez, R.L. and Denhardt, D. T *et al.* (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, Butterworth, Stoneham (1988)).

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The invention also provides plasmids containing gene constructs in which ~~the~~ a gene coding for a malate dehydrogenase and a gene coding for an enzyme ~~for~~ which catalyzes the transformation of an organic substrate ~~with~~ in a reaction accompanied by the consumption

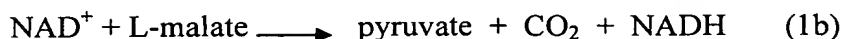
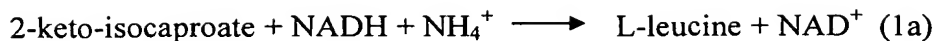
of NAD(P)H are present. Possible plasmids or vectors are well known in the art and of origin are in principle all the embodiments available to the expert for this purpose. Such plasmids and vectors can be found, e.g. e.g., in Studier, *et al.*, and colleagues (Studier, W. F.; Rosenberg A. H.; Dunn J. J.; Dubendorff J. W.; (1990), Use of the T7 RNA polymerase to direct expression of cloned genes, Methods Enzymol. 185, 61-89 Meth. Enzymol. 185:61-89 (1990)) or the brochures of Novagen, Promega, New England Biolabs, Clontech or Gibco BRL. Further preferred plasmids and vectors can be found in: Glover, D. M. (1985), DNA cloning: A Practical Approach, vol. I-III, IRL Press Ltd., Oxford (1985); Rodriguez, R.L. and Denhardt, D. T. *et al.* (eds) (1988), Vectors: a survey of molecular cloning vectors and their uses, 179-204, (1988); Butterworth, Stoneham; Goeddel, D. V. (1990), Systems for heterologous gene expression, Methods Enzymol. 185, 3-7 et al., Meth. Enzymol. 185:3-7 (1990); Sambrook, J.; Fritsch, E. F. and Maniatis, T. (1989) *et al.*, Molecular cloning: a laboratory manual, 2<sup>nd</sup> ed., Cold Spring Harbor Laboratory Press, New York (1989).

Plasmids Preferred plasmids for cloning gene constructs include with which the gene construct containing the nucleic acids according to the invention can be cloned into the a host organism in a very preferred manner are: pUC18 (Roche Biochemicals), pKK-177-3H (Roche Biochemicals), pBTac2 (Roche Biochemicals), pKK223-3 (Amersham Pharmacia Biotech), pKK-233-3 (Stratagene) or and pET (Novagen). The plasmid pkk/phe/mali (fig. 5) is advantageous may be used in this connection. The preparation of this plasmid and of a corresponding rec-microorganism recombinant microorganism is described in the dissertation by S. Naamnieh; (University of Düsseldorf, in preparation p. 70 et seq).

For the use, the The polypeptides in-question of the process according to the invention can be used in the free form as homogeneously purified compounds or as an enzyme prepared by a recombinant method. These polypeptides can furthermore also be employed as a constituent of an intact guest organism or in combination with the broken-down cell mass of the host organism, which has been purified to any desired extent. The use of the enzymes in immobilized form is also possible (Sharma B. P.; Bailey L. F. and Messing R. A. (1982), Immobilisierte Biomaterialien Techniken und Anwendungen, Angew. Chem. 94, 836-852 , et al., Angew Chem. 94:836-852 (1982)). The immobilization is advantageously carried out by lyophilization (Paradkar, V. M.; Dordiek, J. S. (1994), Aqueous-Like Activity of  $\alpha$ -Chymotrypsin Dissolved in Nearly Anhydrous Organic

Solvents, *J. Am. Chem. Soc.* 116, 5009-5010 *et al.*, *J. Am Chem. Soc.* 116:5009-5010 (1994); Mori, T.; Okahata, Y. (1997), A variety of lipi-coated glycoside hydrolases as effective glycosyl transfer catalysts in homogeneous organic solvents, *Tetrahedron Lett.* 38, 1971-1974, *et al.*, *Tetrahedron Lett.* 38:1971-1974 (1997); Otamiri, M.; Adlercreutz, P.; Matthiasson, B. (1992), Complex formation between chymotrypsin and ethyl cellulose as a means to solubilize the enzyme in active form in toluene, *Biocatalysis* 6, 291-305 *et al.*, *Biocatalysis* 6:291-305 (1992)). Lyophilization in the presence of surface-active substances, such as Aerosol OT or polyvinylpyrrolidone or polyethylene glycol (PEG) or Brij 52 (diethylene glycol monocetyl ether) (Kamiya, N.; Okazaki, S. Y.; Goto, M. (1997), Surfactant horseradish peroxidase complex catalytically active in anhydrous benzene, *Biotechnol. Tech.* 11, 375-378 *et al.*, *Biotechnol. Tech.* 11:375-378 (1997)), is very particularly preferred. Immobilization on Eupergit<sup>®</sup>, in particular Eupergit C<sup>®</sup> and Eupergit 250L<sup>®</sup> (Röhm) (Eupergit.RTM. C, a carrier for immobilization of enzymes of industrial potential. Katchalski-Katzir, E.; Kraemer, D. M. *Journal of Molecular Catalysis B: Enzymatic* (2000), 10(1-3), 157-176 *et al.*, *J. Mol. Catal. B: Enzymatic* 10:157-176 (2000)), is extremely still more preferred. Immobilization on Ni-NTA in combination with the polypeptide supplemented with the His tag (hexa-histidine) is likewise preferred (Purification of proteins using polyhistidine affinity tags. Bornhorst, Joshua A.; Falke, Joseph J. *Methods in Enzymology* (2000), 326, 245-254 *et al.*, *Meth. Enzymol.* 326:245-254 (2000)). The use as CLECs is also conceivable possible (St. Clair, N.; Wang, Y. F.; Margolin, A. L. (2000), Cofactor bound cross-linked enzyme crystals (CLEC) of alcohol dehydrogenase, *Angew. Chem. Int. Ed.* 39, 380-383 *et al.*, *Angew Chem. Int. Ed.* 39:380-383 (2000)). By these measures, it can be is possible to generate from polypeptides which become unstable due to organic solvents those which can operate in mixtures of aqueous and organic solvents or entirely in organic media.

The process according to the invention can be carried out such that the MDH from *E. coli* is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH (equation (1)).



The course of the reaction ~~was is~~ monitored by HPLC. ~~The result can be seen from~~  
 5 ~~the following and results are shown in table 1 in along with results from comparison with~~  
 an equivalent conversion that is carried out with FDH instead of MDH.

Table 1: Comparison of the formation of L-leucine (HPLC) in a coupled batch with  
 coenzyme regeneration by MDH and FDH. In each case, 10 mM  
 10 ketoisocaproate is used, and for the regeneration 100 mM L-malate is used  
for regeneration by malate dehydrogenase (MDH) 100 mM L-malate or and  
100 mM formate is used for regeneration by formate dehydrogenase (FDH)  
100 mM formate were employed.

| Time<br>[min] | L-Leucine with MDH<br>[mM] | L-Leucine with FDH<br>[mM] |
|---------------|----------------------------|----------------------------|
| 0             | 0.7                        | 0.1                        |
| 10            | 8.8                        | 9.9                        |
| 30            | 9.6                        | 11.1                       |
| 60            | 10.8                       | 9.8                        |
| 120           | 9.9                        | 9.9                        |

The use of alcohol dehydrogenases in combination with malate dehydrogenases ~~was~~  
 is also ~~investigated possible~~. The MDH from *E. coli* is coupled with an NAD-dependent  
 S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH;  
 DE10218689). The usability of the MDH is tested here via the reduction of a ketone (p-Cl-  
 25 acetophenone = pCAp) in accordance with equation (4).

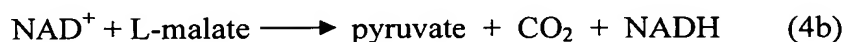
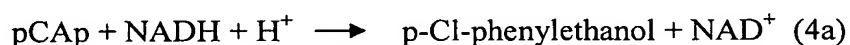


Table 2: Decrease in the ketone p-Cl-acetophenone (employed 10 mM) and increase in the enzymatically formed alcohol p-Cl-phenylethanol (100%, corresp. to 10 mM) as a function of time.

| Time [min] | Ketone [%] | Alcohol [%] |
|------------|------------|-------------|
| 0          | 100        | 0           |
| 10         | 28         | 72          |
| 20         | 12         | 88          |
| 30         | 4          | 96          |
| 60         | 0          | 100         |

5            ~~Enantiomerically~~ The term “enriched” or “enantiomer-enriched” describes the fact that one optical antipode is present in a mixture at with its other to >50%. If one stereo-  
centre is present, the structures shown relate to the two possible enantiomers, and if more  
than one stereo-centre is present in the molecule, they relate to all the possible  
diastereomers and, in respect of a diastereomer, to the two possible enantiomers of the  
10    compound in question ~~which fall under this~~.

The organism *Candida boidinii* is deposited under number ATCC 32195 at the  
American Type Culture Collection and is accessible to the public.

15            The documents of the prior art mentioned in this specification are ~~regarded as also~~  
~~included in the disclosure~~ hereby incorporated by reference herein.

#### ~~Descriptions of the drawings: Brief Description of the Drawings~~

20            ~~Fig. 2 shows a membrane reactor with dead-end filtration. The substrate (1) is~~  
~~transferred via a pump (2) into the reactor space (3), which contains a membrane (5). In the~~  
~~reactor space, which is operated with a stirrer, are, in addition to the solvent, the catalyst~~  
~~(4), the product (6) and unreacted substrate (1). Low molecular weight product (6) is chiefly~~  
~~filtered off via the membrane (5).~~

25            ~~Fig. 3 shows a membrane reactor with cross-flow filtration. The substrate (7) is~~  
~~transferred here via the pump (8) into the stirred reactor space, in which is also solvent,~~  
~~eatalyst (9) and product (14) are also present. A solvent flow which leads via a heat~~

exchanger (12), which may be present, into the cross-flow filtration cell (15) is established via the pump (16). The low molecular weight product (14) is separated off here via the membrane (13). High molecular weight catalyst (9) is then passed back with the solvent flow, if appropriate via a heat exchanger (12) again, if appropriate via the valve (11), into the reactor (10).

Figure 1: Effect of pH on MDH Activity. MDH was isolated from *E. coli* and its activity at different pH values examined. Maximum activity occurred at pH values of 11 and higher. Nevertheless, good activity is still observed at pH values as low as 7.0. The experiment shown in the figure is more fully described in Example 1.

Figure 2: Membrane Reactor with Dead-End Filtration. Substrate (1) is transferred via a pump (2) into the reactor space (3), which contains a membrane (5). In the reactor space, which is operated with a stirrer, are, in addition to the solvent, the catalyst (4), the product (6) and unreacted substrate (1). Low molecular weight product (6) is chiefly filtered off via the membrane (5).

Figure 3: Membrane Reactor with Cross-Flow Filtration. The substrate (7) is transferred by pump (8) into the stirred reactor space, in which solvent, catalyst (9) and product (14) are also present. A solvent flow which leads via a heat exchanger (12), which may be present, into the cross-flow filtration cell (15) is established via the pump (16). The low molecular weight product (14) is separated off here via the membrane (13). High molecular weight catalyst (9) is then passed back with the solvent flow, if appropriate via a heat exchanger (12) again, if appropriate via the valve (11), into the reactor (10).

Figure 4: Effect of Temperature on MDH Activity: The figure shows the results of an experiment examining the effect of temperature on the activity of MDH isolated from *E. coli*. The optimum temperature of the MDH reaction is approximately 55°C.

Figure 5: The Plasmid pkk/phe/mali: The figure is a diagram of an expression vector encoding both phenylalanine dehydrogenase and malate dehydrogenase.

Figure 6: HPLC Analysis of Reaction Product: A whole cell conversion reaction was carried out (see Example 8). The phenylalanine formed in the reaction was assayed by HPLC and results are shown in the figure.

5        Figure 7: Product Yield: The phenylalanine formed in a whole cell conversion reaction (see Example 8) was monitored over a period of 20 hours and results are shown in the figure.

10       Figure 8: Construction of Plasmid containing pheDH and Malate Dehydrogenase: The figure shows diagrammatically the steps used in constructing a plasmid that can be in connection with a whole cell conversion reaction (see Example 4).

15       Figure 9: Optimization of Cell Lysis by Ultrasound: Crude preparations of phenyl alanine dehydrogenase and malate dehydrogenase were prepared by ultrasound (see description in Example 6). Cells were exposed to ultrasound treatment for either 30 or 60 seconds and then cooled for 30 seconds. This treatment was repeated 2-8 times and the amount of pheDH and malate DH activity in the lysate was determined. Results are shown in the figure.

20       Figure 10: Optimum pH of Malate Dehydrogenase and PheDH Reactions. The effect of pH on the activity of pheDH and malate DH was examined and results are shown in the figure. The measurements were carried out with partly purified enzyme and are described in Example 7.

25       Figure 11: Optimum pH of Malate Dehydrogenase and PheDH Reactions. The effect of temperature on enzyme activity was measured and results are shown in the figure. The experiment is described in Example 7.

30       Figure 12: Formation Kinetics for Phenylalanine: A coupled reaction of pheDH and malate DH was carried out as described in Example 7. The formation of phenylalanine was monitored by HPLC over several hours and results are shown in the figure.



## Examples

Example 1: Purification and biochemical properties of the MDH from *E. coli*

For production of the MDH from *E. coli* K12 used here, see the dissertation by S. Naamnich, University of Düsseldorf, in preparation, p. 70 et seq.

### 5 Purification and biochemical properties of the MDH from *E. coli*:

#### a) A Purification

The purification of the recMDH from *E. coli* crude extracts (expression strain: *E. coli* derivative JM105) was carried out in accordance with the purification protocol of Stols et al. (Stols L., and Donnelly M. I. (1997). Production of succinic acid through  
 10 overexpression of NAD(+) dependent malic enzyme in an Escherichia coli mutant. Appl Environ Microbiol **63**: 2695-701. 63:2695-701 (1997)). The rec-bacteria cells were first broken down by disintegration with glass beads (breakdown buffer Tris/HCl 100 mM pH 7.5). Thereafter, a purification step by a Q-Sepharose was carried out. After the purification by means of Q-Sepharose, it was possible to determine a specific activity of the MDH of  
 15 about 7.3 U/mg. By purification of this enzyme by means of further Using chromatography steps, on hydroxyapatite and Phenylsepharose phenylsepharose, it was possible to purify the MDH to homogeneity with a specific activity of 133 U/mg.

Tab. 3: Summary of the purification of the rec-MDH from *E. coli*.

| Purification step | Activity (U) | Protein (mg) | Spec. act. (U/mg) | Enrichment (-fold) | Yield (%) |
|-------------------|--------------|--------------|-------------------|--------------------|-----------|
| Crude extract     | 210          | 202          | 1.03              |                    | 100       |
| Q-Sepharose       | 184          | 25.2         | 7.3               | 7.1                | 88        |
| Hydroxyapatite    | 68           | 1.6          | 42.5              | 41                 | 32        |
| Phenylsepharose   | 24           | 0.18         | 133               | 129                | 11        |

20

#### b) B. Biochemical characterization

##### - Km values

For L-malate a Km value of 0.29 mM was measured, and for the coenzyme NAD<sup>+</sup> a Km value of 0.14 mM. Both Km values lie in a low range of < 1 mM, and they show  
 25 suggesting that the two substrates are recognized by the enzyme with a good affinity. The two values suggest that the MDH can be used for the regeneration of NADH.

- Optimum pH (fig. 1)

MDH shows a maximum activity at relatively high pH values of 11 and higher. Nevertheless, the drop in activity at lower pH values is relatively small, thus 72% activity is still present at pH 8.0, and 67% activity ~~still~~ is still present at pH 7.0.

5

- Optimum temperature

The optimum temperature of the MDH reaction is approx. 55°C (fig. 4).

Example 2: Coupling Reactions

10

a) A. Coupling of leucine dehydrogenase with malate dehydrogenase

The MDH from *E. coli* is coupled with an NAD-dependent leucine dehydrogenase (LeuDH from *Bacillus cereus*; Sigma). LeuDH catalyses the reductive amination of aliphatic keto acids, such as, for example, ketoisocaproate, to the corresponding L-amino acids, such as L-leucine, with consumption of NADH.

15

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses): 526 µl Hepes buffer (200 mM Hepes, pH 8.5 with 10 mM MgCl<sub>2</sub>); 143 µl ammonium sulfate solution (500 mM in the test); 100 µl ketoisocaproate (100 mM); 20 µl NAD<sup>+</sup> (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 1 µl LeuDH (0.5 U in the test); 10 µl MDH (1.5 U in the test; partly purified). The test batch is incubated at 30°C, and after 0, 10, 30, 60 and 120 min samples are taken (50 µl; Eppendorf reaction vessels) and heated for 3 min at 95°C to stop the reaction. Denatured protein is separated off by centrifugation for 10 min at 13,000 rpm (Eppendorf bench centrifuge) and the supernatant is analysed by means of HPLC, after  
25 derivatization with ortho-phthalaldehyde (OPA).

Derivatization with OPA (= ortho-phthaldialdehyde):

140 µl Na borate buffer (100 mM; pH 10.4); 40 µl sample or standard; 20 µl OPA/IBLC reagent (= ortho-phthaldialdehyde / N-isobutyryl-L-cysteine). 20 µl of this  
30 reaction solution are injected for the HPLC analysis.

HPLC analysis: The results of HPLC analyses are shown in table 1.

~~For the results see table 1:~~

b) B. Comparison experiment: Coupling of leucine dehydrogenase with formate dehydrogenase

In a comparison batch carried out in parallel, the same components as described above were used, but instead of the malate dehydrogenase, 0.5 U formate dehydrogenase (FDH from *Candida boidinii*; Sigma) was employed and instead of 100 mM L-malate 100 mM formate was used as the regeneration substrate. Results are shown in table 1.

~~For the results see table 1.~~

Example 3: Coupling of the MDH with alcohol dehydrogenase:

The MDH from *E. coli* (expression strain: *E. coli* derivative JM 105) is coupled with an NAD-dependent S-specific alcohol dehydrogenase from *Rhodococcus erythropolis* (RE-ADH). The usability of the MDH is tested ~~here~~ via the reduction of a ketone (p-Cl-acetophenone).

Test batch (1 ml in total; unless stated otherwise the concentration of the stock solution is stated in parentheses): 678.7 µl Hepes buffer (100 mM Hepes, pH 8.5 with 10 mM MgCl<sub>2</sub>); 1.3 µl p-Cl-acetophenone (10 mM in the test); 20 µl NAD<sup>+</sup> (50 mM); 200 µl L-malate (500 mM, Na salt, dissolved in Hepes buffer, pH 8.5); 15 µl RE-ADH (1 U in the test); 85 µl MDH (1 U in the test; partly purified).

The test batch is incubated at 30°C, and after 0, 10, 20, 30 and 60 min samples (50 µl) are taken, 100 µl ethyl acetate are added and the upper phase is analysed by means of gas chromatography for the formation of the alcohol p-Cl-phenylethanol. ~~For the results see~~ Results are shown in table 2.

Example 4: Construction of an expression vector with heterologous expression

From the sequence of the amplified fragment, primers with integrated restriction cleavage sites and a codon for the ribosomal binding site were constructed. After amplification of the malate dehydrogenase from the recombinant pUC18, the PCR fragment was cloned into the recombinant recPhe-pKK-223-3 expression vector after the PheDH sequence at the PstI and HindIII restriction cleavage sites (fig. 8 – Construction of the plasmid for a heterologous expression for L-Phe synthesis by means of whole cell conversion).

PCR:

5' forward: N'-malic-pst

5' CTGCAGAGCCCAGGGATGGATATTCAAAAA 3'

concentration 100 pmol/μl

5

5' reverse: C'-malic-Hin

5' AAGCTTTTAGATGGAGGTACGGCGGTAGTC 3'

concentration 100 pmol/μl

10 Table 4: PCR protocol for amplification of the malate dehydrogenase from the recombinant pUC18 plasmid. The concentrations of the template DNA were varied.

| Template<br>DANN<br>recpUC18 | N'-malic-pst<br>prim 1 | C'-malic-Hin<br>prim 2 | dNTP | Buffer | Taq<br>polymerase | H <sub>2</sub> O |
|------------------------------|------------------------|------------------------|------|--------|-------------------|------------------|
| 50 ng/μl                     | 1 μl                   | 1 μl                   | 2 μl | 10 μl  | 1 μl              | 83 μl            |
| 25 ng/μl                     | 1 μl                   | 1 μl                   | 2 μl | 10 μl  | 1 μl              | 83 μl            |
| 10 ng/μl                     | 1 μl                   | 1 μl                   | 2 μl | 10 μl  | 1 μl              | 83 μl            |

One cycle consists of:

Denaturing step : 94°C

Annealing step: 59°C

15 Amplification step: 72°C

Cloning:

20 The new construct of the recombinant plasmid (fig. 5) was transformed into competent *E. coli* cells JM 105 or HB 101. The standard transformation was carried out in accordance with the protocol of Hanahan (Hanahan, D. (1983). ~~Studies on transformation of~~ *E. coli* with plasmids. *J Mol Biol* ~~166: 557-80.~~ 166:557-580 (1983)). For this, 100-200 μl of competent *E. coli* cells were thawed on ice and 40 ng of DNA from the ligation batch were added. The plasmid cell suspension was cooled for 30 min on ice and then heated at 42°C for 90 sec and immediately cooled again on ice. After addition of 300 μl LB medium, the cells were incubated for about 45 min at 37°C for regeneration. 200 μl of this culture were then plated out on to an LB plate containing antibiotic and incubated overnight at 37°C.

With the cloning of the malate dehydrogenase 3' to the PheDH on at the PstI and HindIII cleavage site with its own ribosomal binding site, expression of the two enzymes could take place simultaneously. ~~Note: Further detailed information on the experimental preparation of these cells will be described in: S. Naamnieh, *Dissertation*, University of~~  
 5 ~~Düsseldorf, in preparation.~~

#### Example 5: Coexpression of PheDH and malate dehydrogenase

~~Of the positive clones, some were selected for expression~~ Certain positive clones were selected for expression. An individual colony of ~~the particular clones~~ a clone was  
 10 transinoculated into 5 ml LB<sub>amp</sub> medium, and after OD<sub>580</sub> = 0.6 was reached, ~~was~~ cells were induced with 1 mM IPTG. The induction was carried out overnight and the harvested cells were broken down with ultrasound.

The recombinant strain HB101 shows a malate dehydrogenase activity of 100 U/ml  
 15 and likewise a PheDH activity of 130 U/ml. The activity of the two enzymes in the recombinant strain JM 105 is clearly higher and is at ~ 600 U/ml for malate dehydrogenase and ~ 1,200 U/ml for the PheDH. The two recombinant strains were cultured in a 10 l fermenter and the activity of the two enzymes was determined. Results are shown in Table  
 20 5.

Table 5: Determination of the activity of the expressed enzymes in a 10 l fermenter with LB medium as batch fermentation.

|               | Activity of PheDH |        | Activity of malate dehydrogenase |        |
|---------------|-------------------|--------|----------------------------------|--------|
|               | (U/ml)            | (U/mg) | (U/ml)                           | (U/mg) |
| E. coli HB101 | 400               | 33     | 220                              | 22     |
| E. coli JM105 | 1300              | 118    | 640                              | 71     |

It can be seen from the expression data that the *E. coli* strain JM 105 shows a  
 25 significantly better activity for both enzymes, and all further experiments were therefore carried out with this strain.

### Example 6: Optimization of the activity

To be able to utilize the maximum activity of the crude extracts obtained, several parameters were investigated and varied. The two heterologously expressed enzymes show the best stability properties under different conditions. It was of interest to determine the optimum properties for both enzymes in the same system. The following experiments were therefore carried out ~~with a view to this fact~~.

#### a) A. Optimization of the breakdown buffer

1 g of ~~cells~~ JM<sub>105</sub> cells was broken down in 0.1 M Tris or 0.1 M Kpi buffer with/without BSA (1.5 g/l) 30%. ~~The breakdown~~ Lysis was carried out with ultrasound over 70 cont. cycles. In addition to the buffers, 1.5 g/l BSA were added for stabilization of the enzymes.

Table 6: Comparison of the activity as a function of the breakdown buffer

|                      | 0.1 M Tris |          | 0.1 M Kpi |           |
|----------------------|------------|----------|-----------|-----------|
|                      | - BSA      | + BSA    | - BSA     | + BSA     |
| PheDH                | 520 U/ml   | 610 U/ml | 730 U/ml  | 1100 U/ml |
| Malate dehydrogenase | 430 U/ml   | 720 U/ml | 320 U/ml  | 610 U/ml  |

The addition of BSA led to an increase in the activity in both cases. The breakdown buffer also influenced the activities. It was to be seen here that the suitable breakdown buffer was different for the individual enzymes. The Kpi buffer was more suitable for the PheDH than for the malate dehydrogenase, but since the decrease in activity of the malate dehydrogenase in the Kpi buffer was relatively low, the recombinant cells continued to be broken down in this buffer after the heterologous expression.

#### b) B. Duration of ~~breakdown~~ lysis to ~~investigate the~~ maximize stability

The duration of the breakdown was also investigated and the critical point for stability of the enzymes during this operation was determined. The optimum duration of breakdown can be obtained from these data (fig. 9 – Stability determination of the PheDH and the malate dehydrogenase after various ~~breakdown~~ lysis times by means of ultrasound). The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

The following suspension was used for this experiment:

1 g recombinant cells (JM105)  
3 ml Kpi buffer 0.1 M

During longer treatment of the cells with ultrasound, the activity of the PheDH decreases drastically, whereas the activity of the malate dehydrogenase is retained. The ideal breakdown conditions for a 25% breakdown of 1 g of recombinant *E. coli* JM105 are therefore 4 x 30 s ultrasound treatment with 3 x 30 s intermediate cooling in an ice-bath. During longer treatments with ultrasound, the sample is becomes heated, which can lead to denaturing of the enzymes. The amount of protein was determined in this experiment and can be seen from in the following.

Table 7: Protein determination of the two enzymes expressed, PheDH and malate dehydrogenase, after variation of the breakdown time. The cells were cooled intermediately for 30 seconds after a 60 and 30 second treatment.

|                      | 60 sec x 2<br>spec. activity: U/mg | 30 sec x 4<br>spec. activity: U/mg | 30 sec x 8<br>spec. activity: U/mg |
|----------------------|------------------------------------|------------------------------------|------------------------------------|
| PheDH                | 30                                 | 105                                | 16                                 |
| Malate dehydrogenase | 20                                 | 90                                 | 110                                |

With a purification of the malate dehydrogenase to homogeneity, it was possible to achieve a specific activity of 466 U/mg. The purification steps are summarized in table 8.

Table 8: Purification of the recombinant malate dehydrogenase

|                     | Volume (ml) | Activity (U) | Protein (mg) | Spec. activity (U/mg) | Yield (%) | Factor |
|---------------------|-------------|--------------|--------------|-----------------------|-----------|--------|
| Ultracentrifugation | 0.6         | 820          | 13           | 63                    | 100       | 1      |
| Hydroxy-apatite     | 9           | 470          | 5            | 94                    | 57        | 1.49   |
| Q-Sepharose         | 7           | 496          | 2.1          | 236                   | 60        | 3.74   |
| Phenyl-sepharose    | 1.9         | 280          | 0.6          | 466                   | 34        | 7.39   |

e) C.  $K_M$  value determination

The  $K_M$  values were determined for the substrate and the coenzyme of the malate dehydrogenase. The  $K_M$  values were determined on homogeneous or partly purified recombinant malate dehydrogenase samples.

5        L-Malate:      0.29 mM  
           NAD<sup>+</sup>        0.14 mM

Example 7: Coupled L-phenylalanine synthesis with regeneration of the coenzyme NADH

10        Important factors for a coupled reaction are the optimum pH; ~~and~~ the heat stability of the two enzymes; ~~-In addition to the stability of the enzymes, further factors play a role, and other factors such as, e.g. e.g., the influence of the various substrates on the enzymes. In respect of the~~ With respect to optimum pH, a kinetics study was conducted and the pH-dependency of the synthesis was determined. The increase in the activity at an increasing pH can be seen from fig. 10, ~~a pH of 8.0, in at which although.~~ Although the two enzymes do not show the highest activity at a pH of 8.0, the coenzyme remains stable, being this pH was chosen for the synthesis for coenzyme stability reasons (~~fig. 10 — Optimum pH of malate dehydrogenase and PheDH. The activity of the two enzymes increases as the pH increases. The measurements were carried out with partly purified enzyme. For the~~ phenylalanine dehydrogenase, the reductive amination was measured).

20

A second important factor for the coupled enzyme reaction is the ~~suitable~~ temperature at which the two enzymes remain stable for a relatively long period of time. A further experiment was therefore carried out to determine the optimum ~~temperature (fig. 11 — Optimum temperature. The measurements were carried out at pH 8.5 and in 0.1 M HEPES buffer).~~ As can be seen from fig. 11, the optimum temperature of both enzymes is 50°C. At 30°C the activity measured is only 60%.

25

The malate dehydrogenase is stable at 45°C for a relatively long period of time. However, since the PheDH becomes unstable at this temperature ~~value~~, the syntheses were carried out at 30°C, so that it was possible to ensure the stability of both enzymes and of the coenzyme over a relatively long period of time.

30



Enzymes achieve their optimum activity in the particular suitable buffer. The two enzymes were tested with two different buffers in each case in a reaction batch (table 9). Results are shown in Table 9.

5 Table 9: Comparison of the PheDH and malate dehydrogenase activity in various reaction buffers. The activities are to be seen in per cent of the optimum.

|       | 0.1 M HEPES buffer<br>(pH 8.0) | 0.1 M Tris buffer<br>(pH 8.0) |
|-------|--------------------------------|-------------------------------|
| PheDH | 84 %                           | 100 %                         |
| MDH   | 100 %                          | 42 %                          |

Since the activity of the PheDH in HEPES buffer does not decrease considerably substantially, the coupled enzyme reaction was carried out in this buffer. With the determination of the buffer, pH and temperature values, it was possible to select suitable conditions and media for the synthesis of phenylalanine by a coupled enzyme reaction with regeneration of the cofactor (NADH). 30 mM phenyl pyruvate, 100 mM ammonium sulfate, 100 mM HEPES buffer, 70 mM L-malate, 2 mM NAD<sup>+</sup>, 2 mM Mg<sup>2+</sup>, 25 U PheDH (partly purified) and 30 U malate dehydrogenase were employed. The samples are analysed by means of HPLC. The synthesis was monitored for several hours. After 4 h approx. 50% of the substrate employed, phenyl pyruvate, was converted into L-phenylalanine (fig. 12 – Formation kinetics for L-Phe. The formation of L-Phe was carried out *in situ*).

#### Example 8: Whole cell conversion

20 The following medium was used as the standard batch for the whole cell conversion:

|         |                       |
|---------|-----------------------|
| 0.1 M   | HEPES buffer (pH 8.0) |
| 40 mM   | phenyl pyruvate       |
| 0.1 M   | L-malate              |
| 0.1 M   | ammonium sulfate      |
| 25 2 mM | MgCl <sub>2</sub>     |

The conversion was carried out at 30°C and with 1 g of recombinant *E. coli* cells. The phenylalanine formed was detected by means of HPLC (fig. 6). The formation of L-Phe by recombinant *E. coli* cells was monitored for 20 h and the yield was determined (fig. 7).

No metabolization of the product formed, L-Phe, ~~was to be~~ is detected after incubation for 20 h.

Patent claims: **What is Claimed is:**

1. Process for the preparation of enantiomerically enriched organic compounds in a coupled enzymatic reaction system, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO<sub>2</sub>, characterized in that the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.
2. Process according to claim 1, characterized in that the first enzymatic transformation proceeds using an alcohol dehydrogenase or amino acid dehydrogenase.
3. Process according to claim 2, characterized in that an ADH from *Lactobacillus kefir* or *Rhodococcus erythropolis* is used as the alcohol dehydrogenase and a leucine dehydrogenase or phenylalanine dehydrogenase is used as the amino acid dehydrogenase.
4. Process according to one or more of the preceding claims characterized in that a malate dehydrogenase from *E. coli*, in particular *E. coli* K12, is used.
5. Process according to one or more of the preceding claims characterized in that the reaction is carried out in an aqueous single- or multi-phase solvent mixture.
6. Process according to one or more of the preceding claims, characterized in that the temperature during the reaction is between 20 and 40°C.
7. Process according to one or more of the preceding claims, characterized in that the pH during the reaction is between 6 and 9.
8. Coupled enzymatic reaction system for the preparation of enantiomerically enriched organic compounds, comprising a first enzymatic transformation of an organic substrate, NAD(P)H being consumed, and the regeneration of the NAD(P)H in a second enzymatic transformation by a malate dehydrogenase, with oxidation of L-

malic acid to pyruvate and CO<sub>2</sub>, characterized in that the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.

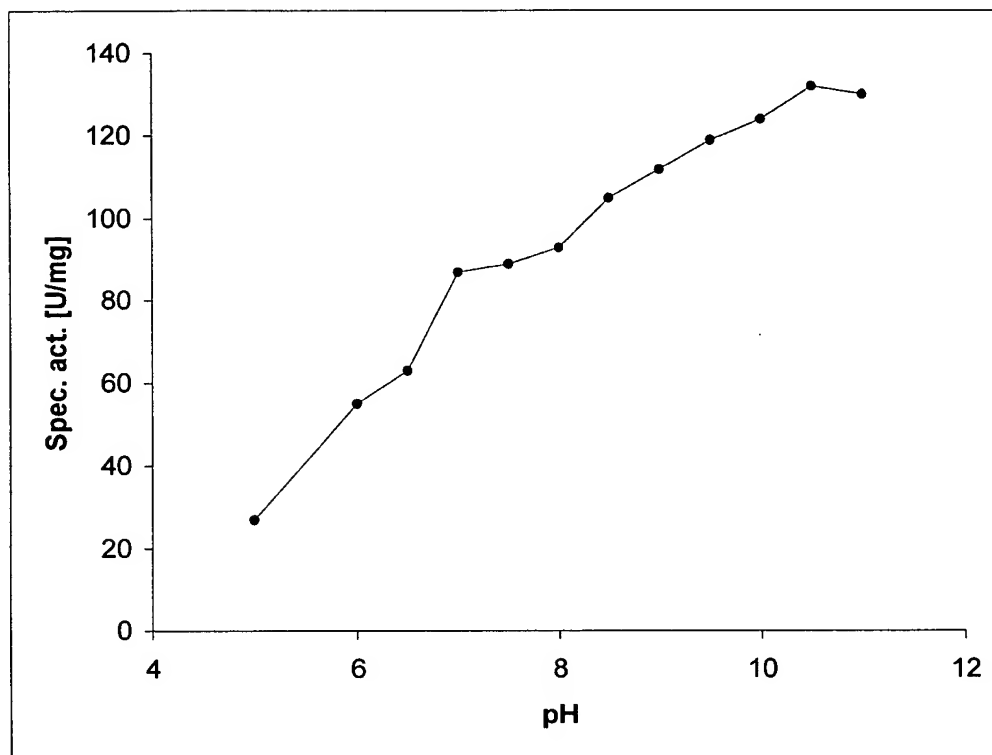
- 5    9.    Whole cell catalyst comprising a cloned gene for a first enzyme for transformation of an organic substrate and a cloned gene for a malate dehydrogenase, this being capable of preparation of an enantiomerically enriched organic compound in a first enzymatic transformation, NAD(P)H being consumed, and of allowing the regeneration of the NAD(P)H to take place in a second enzymatic transformation by
- 10    malate dehydrogenase, with oxidation of L-malic acid to pyruvate and CO<sub>2</sub>, wherein the pyruvate formed from the second enzymatic transformation is not employed as the substrate in the first enzymatic transformation.
- 15    10.    Plasmids containing gene constructs in which the gene for a malate dehydrogenase and a gene for an enzyme for transformation of an organic substrate with consumption of NAD(P)H are present.

~~Abstract:~~

### **Abstract**

The present invention relates to a process for the preparation of enantiomerically enriched organic compounds. In particular, the present invention relates to an ~~enzymatically operating~~ enzymatic process, in which, in a coupled enzymatic reaction system, NAD(P)H is consumed by one enzyme for the preparation of the organic compound and the NAD(P)H is simultaneously regenerated by a second ~~enzyme system~~ enzymatic reaction. A reaction system which operates according to the invention in this manner and ~~an advantageous a~~ whole cell catalyst are also proposed.

Fig. 1:

**Figure 1**

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Fig. 2:

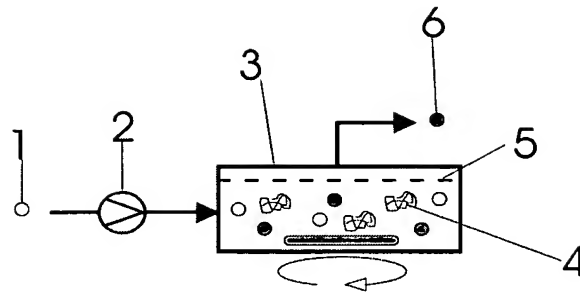
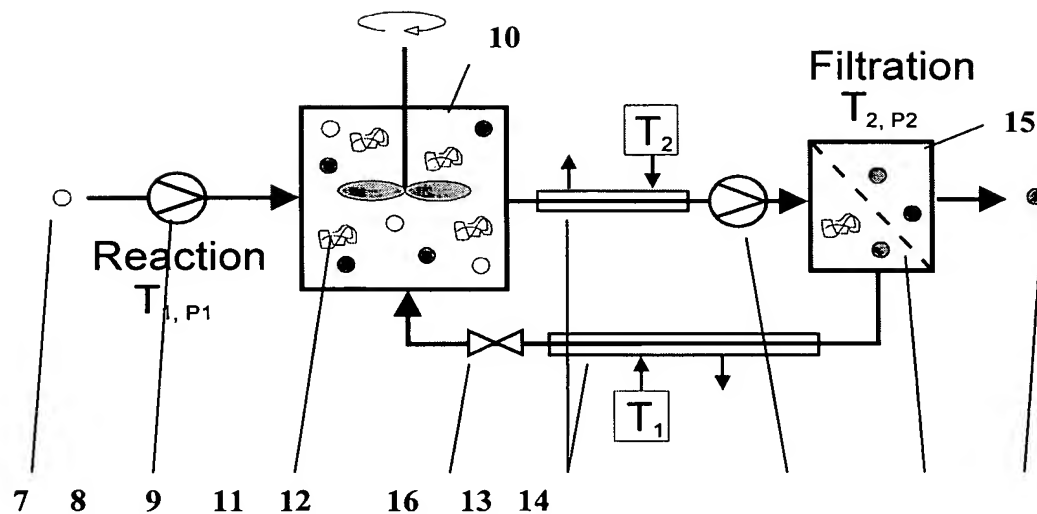
**Figure 2**

Fig. 3:

**Figure 3**

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Fig. 4:

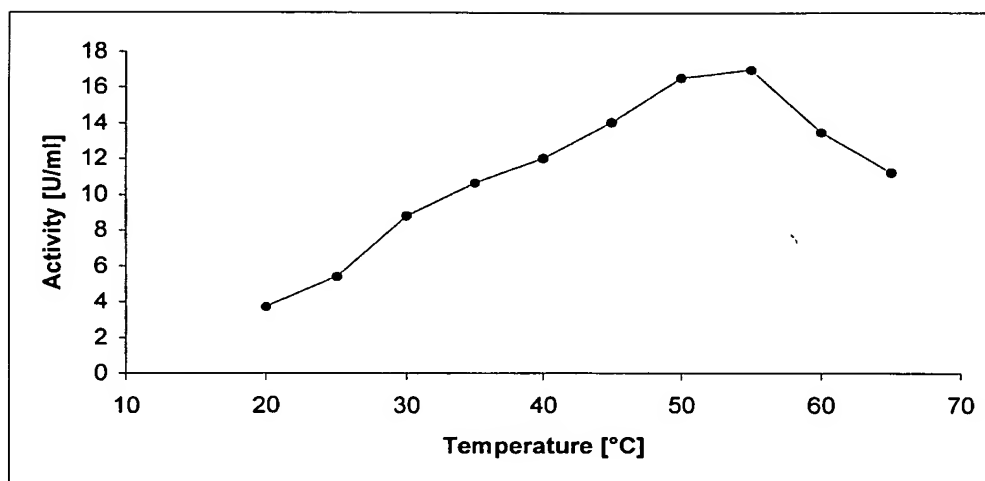
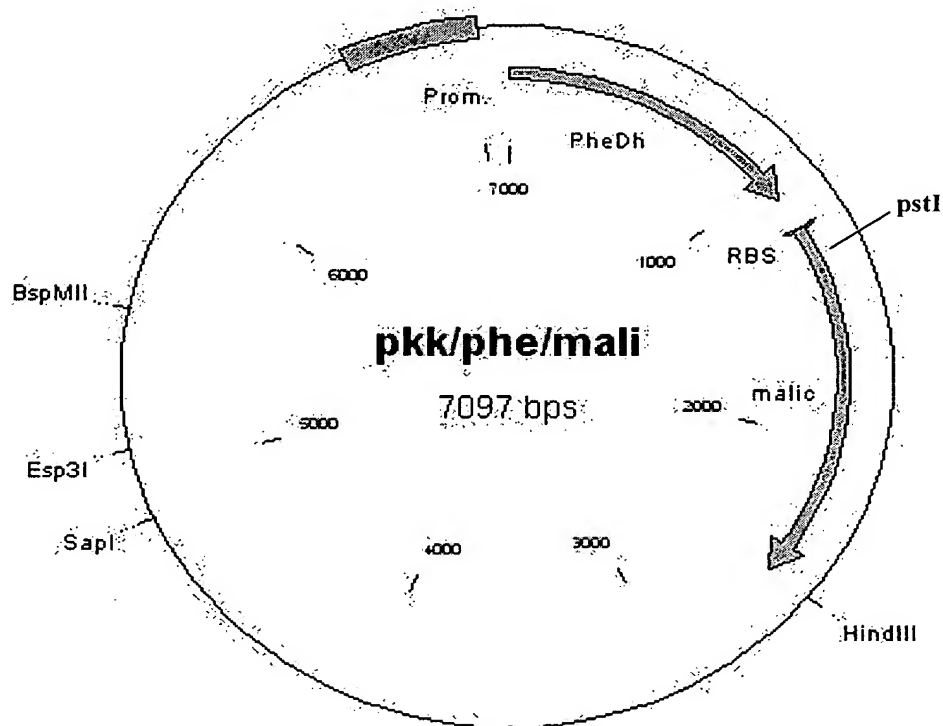
**Figure 4**

Fig. 5:

**Figure 5**



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Fig. 6:

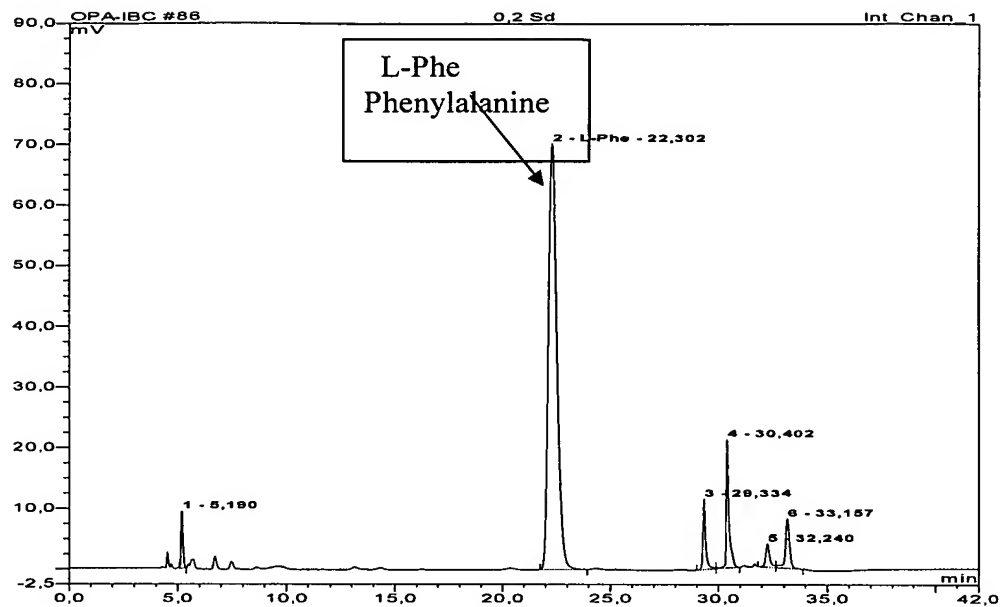
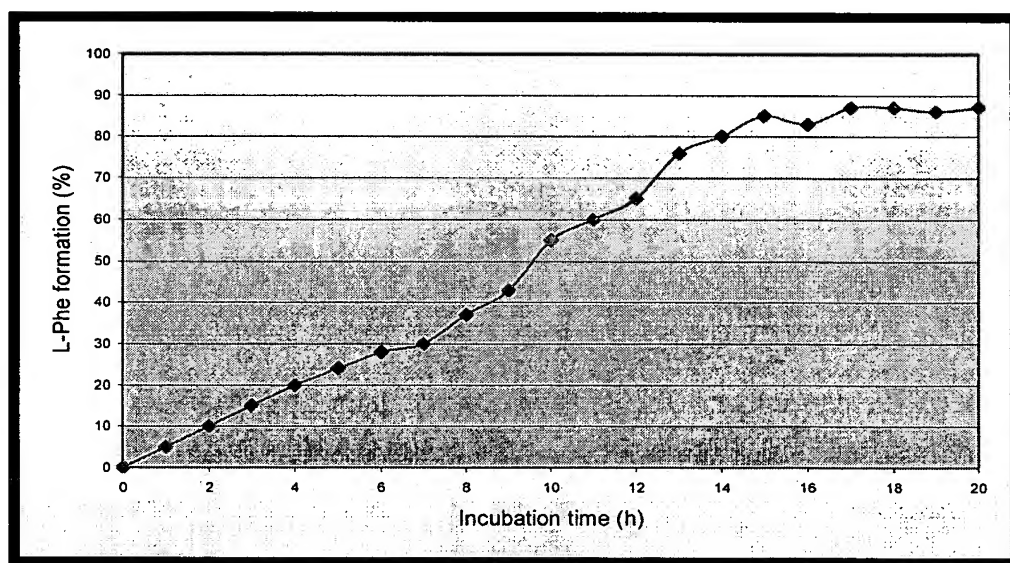
**Figure 6**

Fig. 7:

**Figure 7**

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Fig. 8:

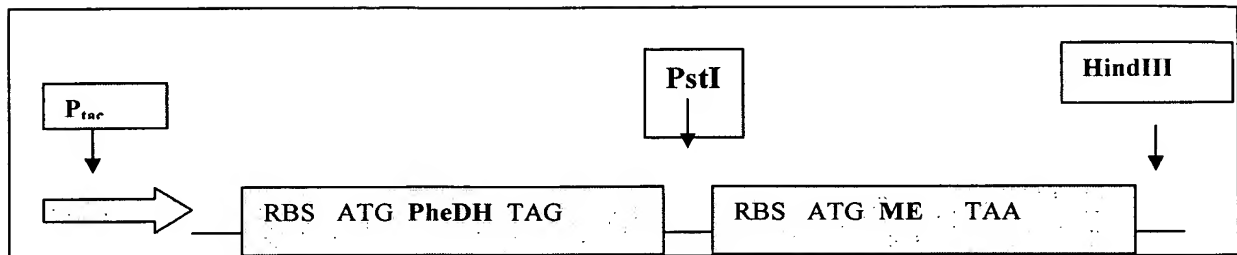
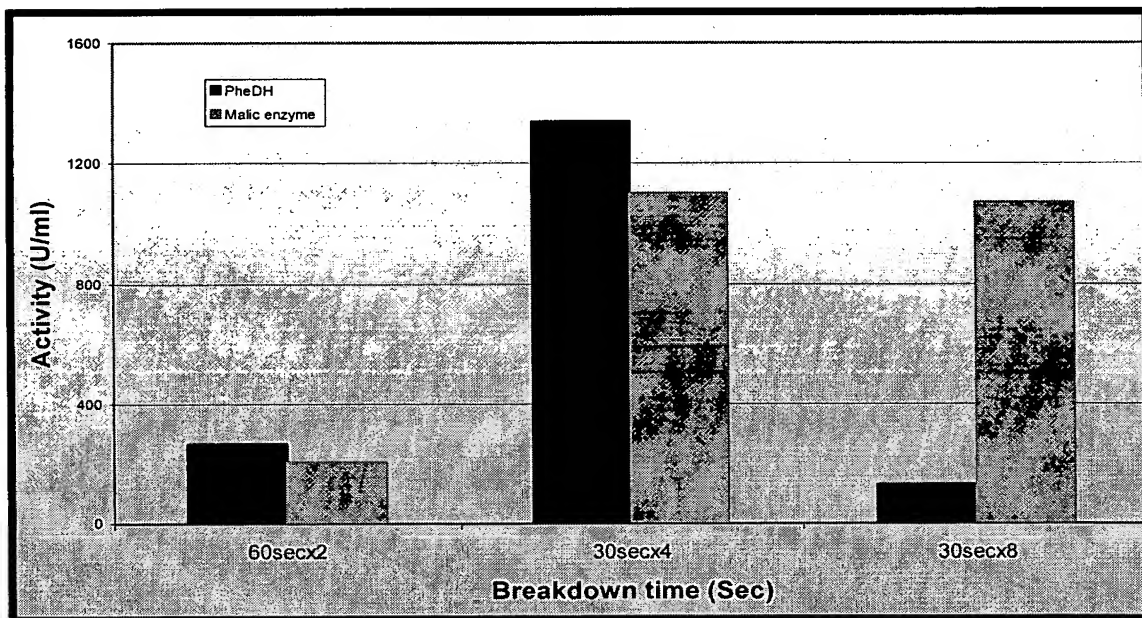
**Figure 8**

Fig. 9:

**Figure 9**

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Fig. 10

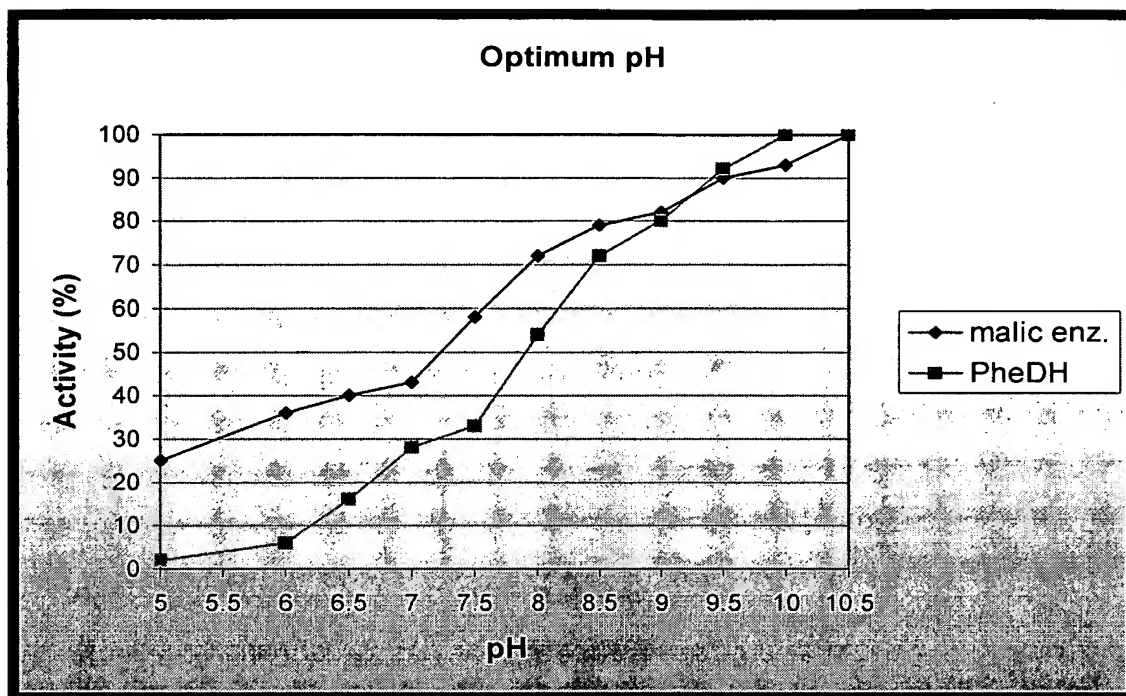
**Figure 10**

Fig-11:

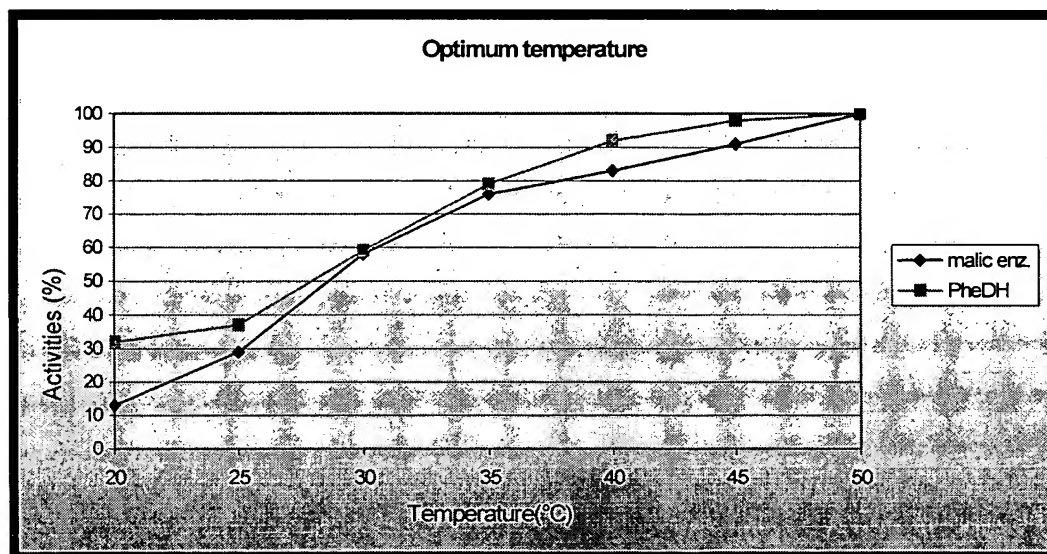
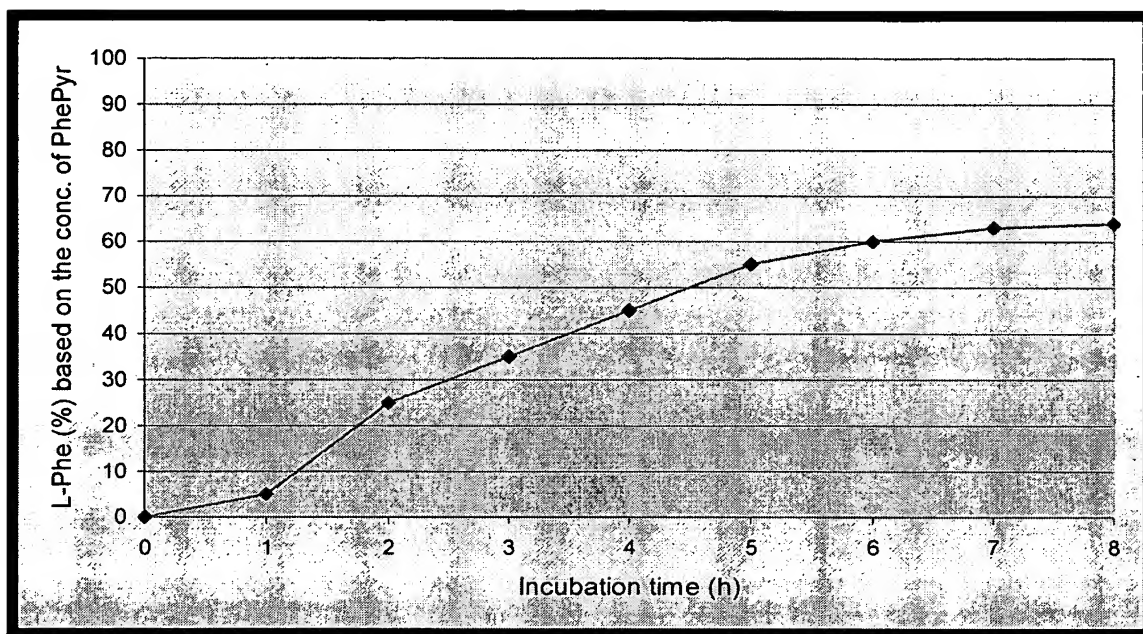
**Figure 11**

Fig. 12

**Figure 12**